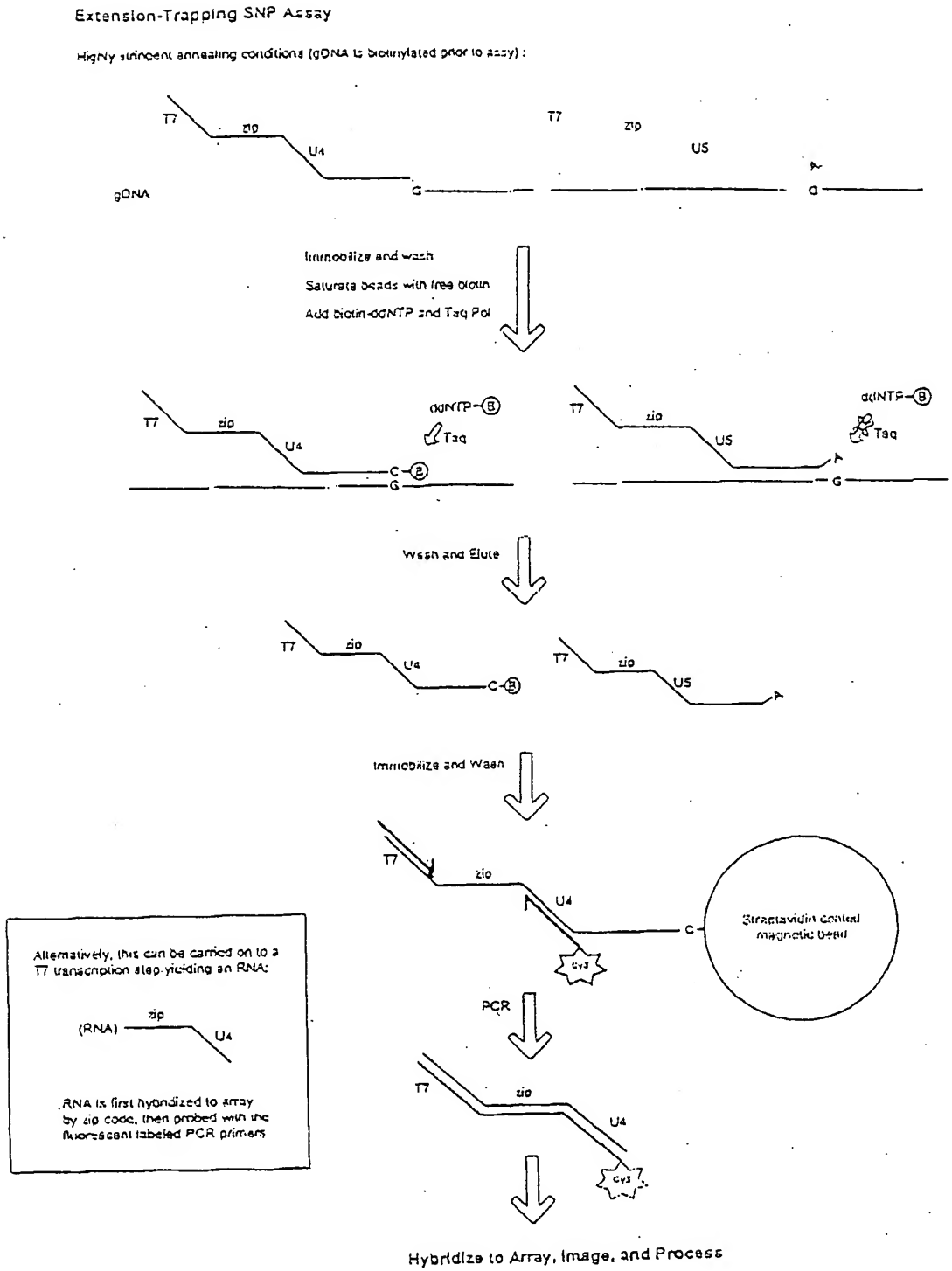

Extension-Trapping SNP Assay
HighY stringent annealing conditions (gDNA is biotinylated prior to assay):
T7
zip
U4
gDNA
G
T7
zip
U5
A
G
Immobilize and wash
Saturate beads with free biotin
Add Biotin-ddNTP and Taq Pol
T7
zip
U4
ddNTP-B
Taq
C-B
G
T7
zip
U5
ddNTP-B
Taq
A
G
Wash and Elute
T7
zip
U4
C-B
T7
zip
U5
A
Immobilize and Wash
T7
zip
U4
C
Cy3
Streptavidin coated magnetic bead
PCR
T7
zip
U4
Cy3
Hybridize to Array, Image, and Process
Alternatively, this can be carried on to a T7 transcription step yielding an RNA:
(RNA)
zip
U4
RNA is first hybridized to array by zip code, then probed with the fluorescent labeled PCR primers

FIGURE 1

Reduced GenomeSingle Base Extension Assay:

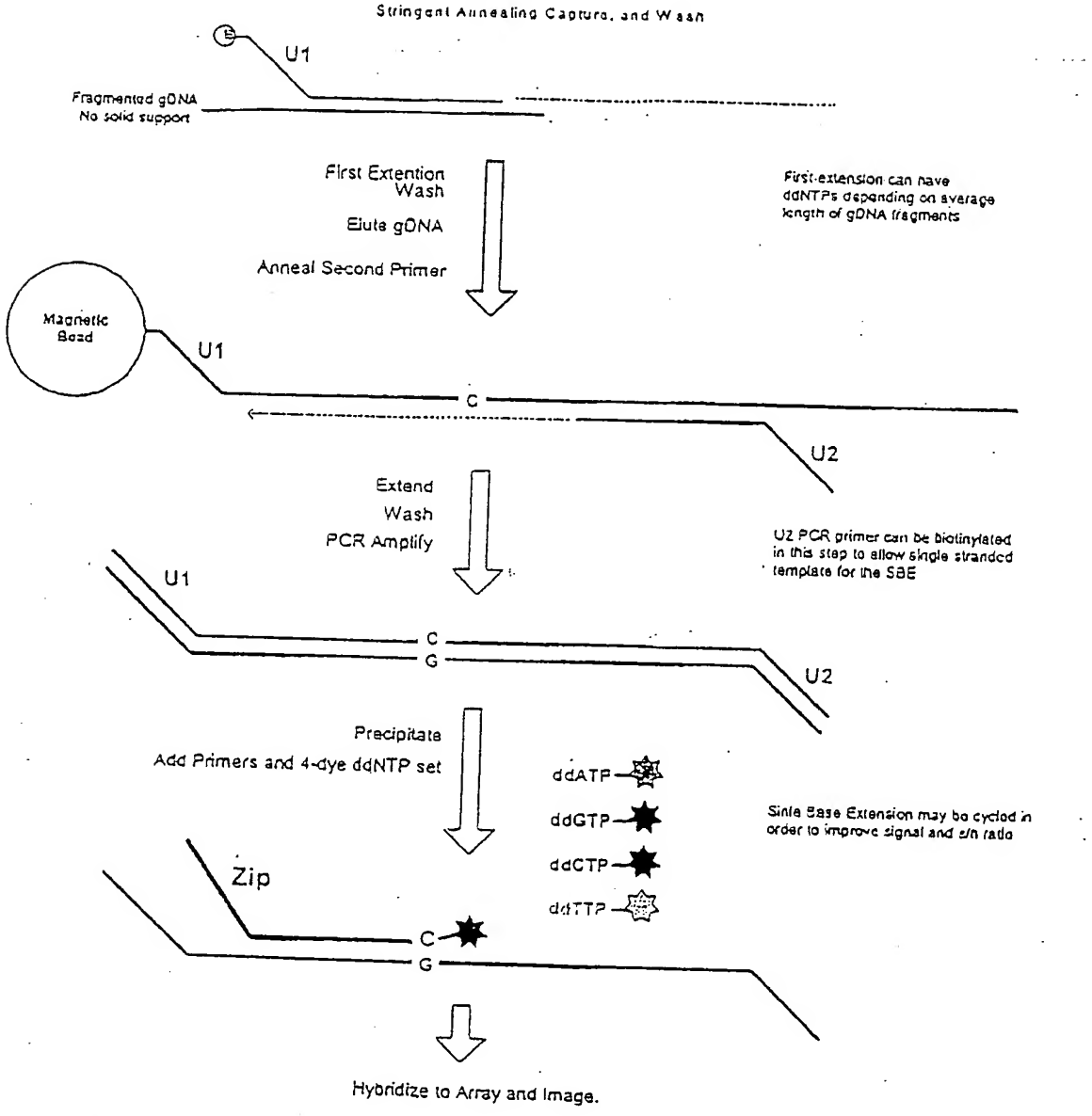


FIGURE 2

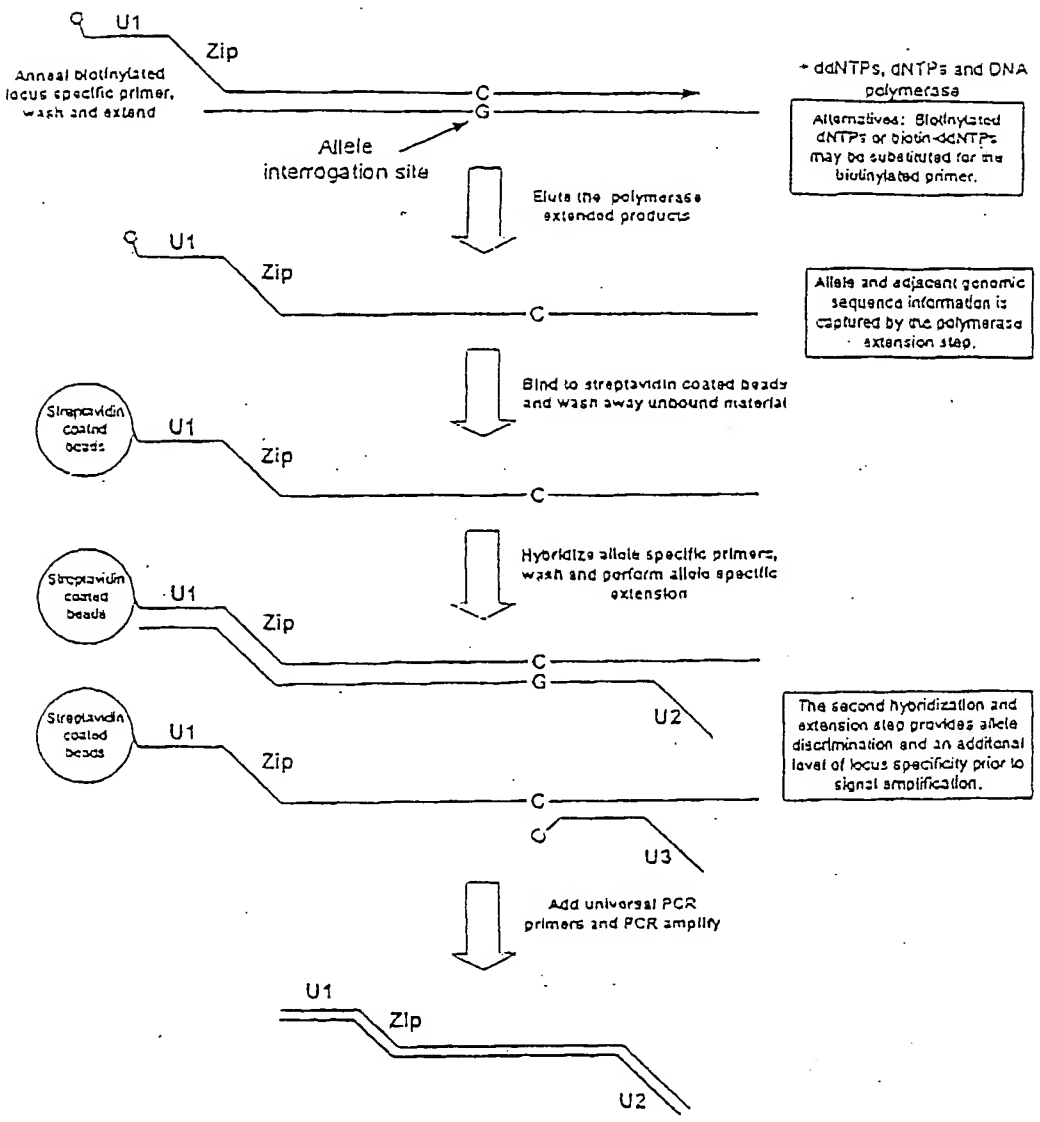

Complexity Reduction and Multiplex Assay
U1
Zip
Anneal biotinylated locus specific primer, wash and extend
+ ddNTPs, dNTPs and DNA polymerase
Alternatives: Biotinylated dNTPs or biotin-ddNTPs may be substituted for the biotinylated primer.
C
G
Allele interrogation site
Elute the polymerase extended products
Allele and adjacent genomic sequence information is captured by the polymerase extension step.
Bind to streptavidin coated beads and wash away unbound material
Streptavidin coated beads
Hybridize allele specific primers, wash and perform allele specific extension
U2
The second hybridization and extension step provides allele discrimination and an additional level of locus specificity prior to signal amplification.
U3
Add universal PCR primers and PCR amplify

FIGURE 3

Complexity Reduction and Multiplex Assay

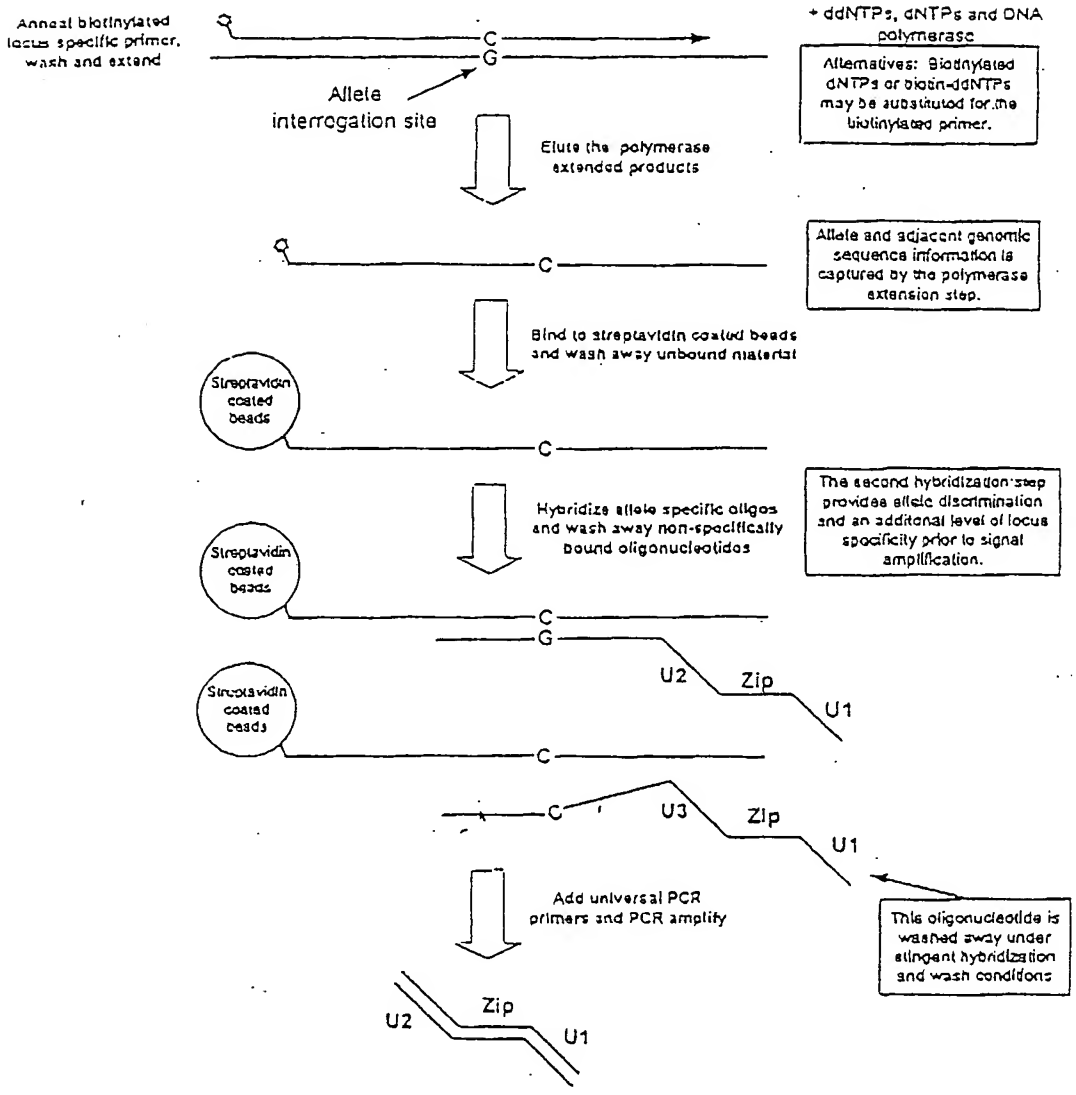


FIGURE 4

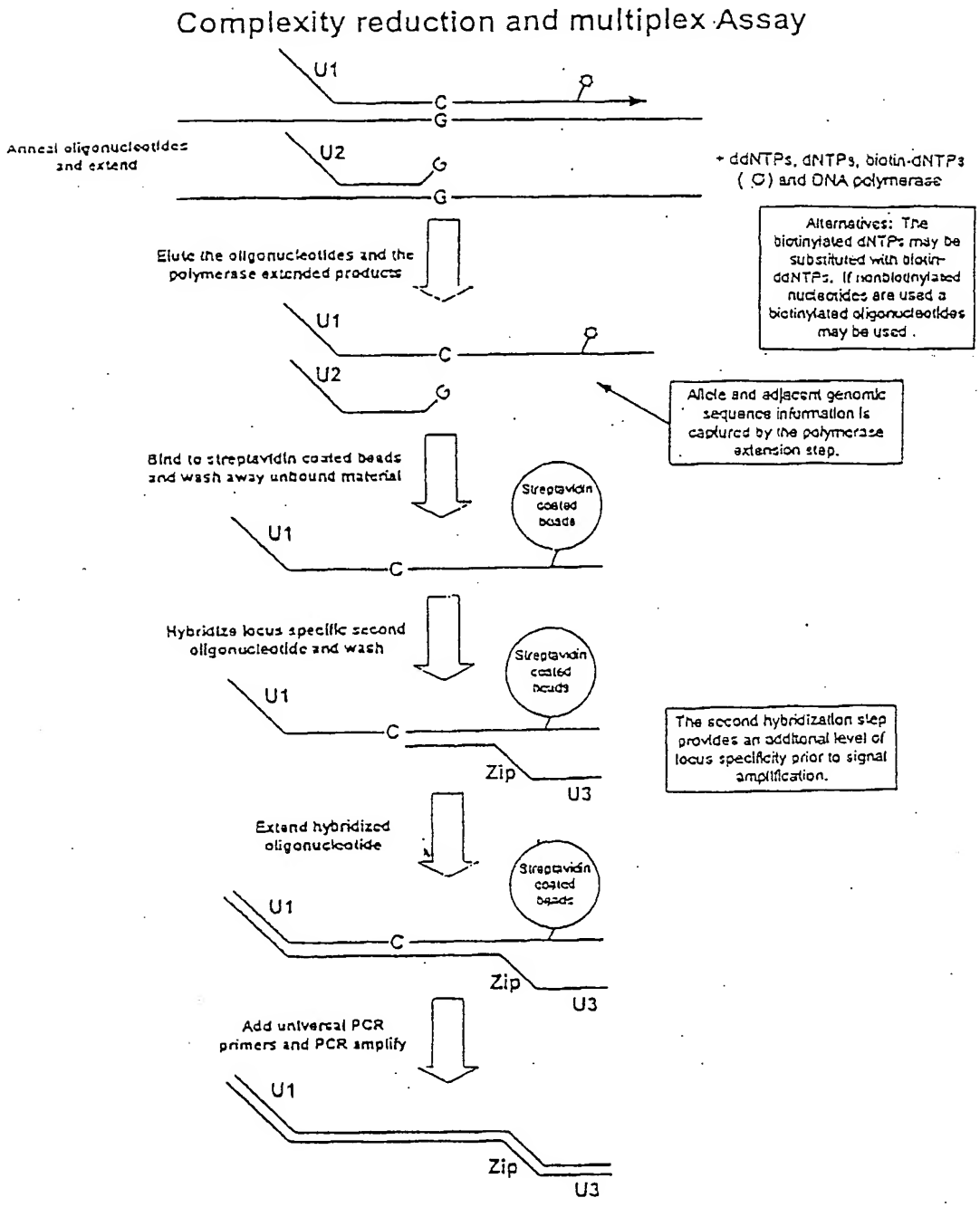

Complexity reduction and multiplex Assay
U1
C
G
Anneal oligonucleotides and extend
U2
G
G
+ ddNTPs, dNTPs, biotin-dNTPs (C) and DNA polymerase
Alternatives: The biotinylated dNTPs may be substituted with biotin-ddNTPs. If nonbiotinylated nucleotides are used a biotinylated oligonucleotides may be used .
Elute the oligonucleotides and the polymerase extended products
U1
C
U2
G
Allele and adjacent genomic sequence information is captured by the polymerase extension step.
Bind to streptavidin coated beads and wash away unbound material
Streptavidin coated beads
U1
C
Hybridize locus specific second oligonucleotide and wash
Streptavidin coated beads
U1
C
Zip
U3
The second hybridization step provides an additional level of locus specificity prior to signal amplification.
Extend hybridized oligonucleotide
Streptavidin coated beads
U1
C
Zip
U3
Add universal PCR primers and PCR amplify
U1
Zip
U3

FIGURE 5

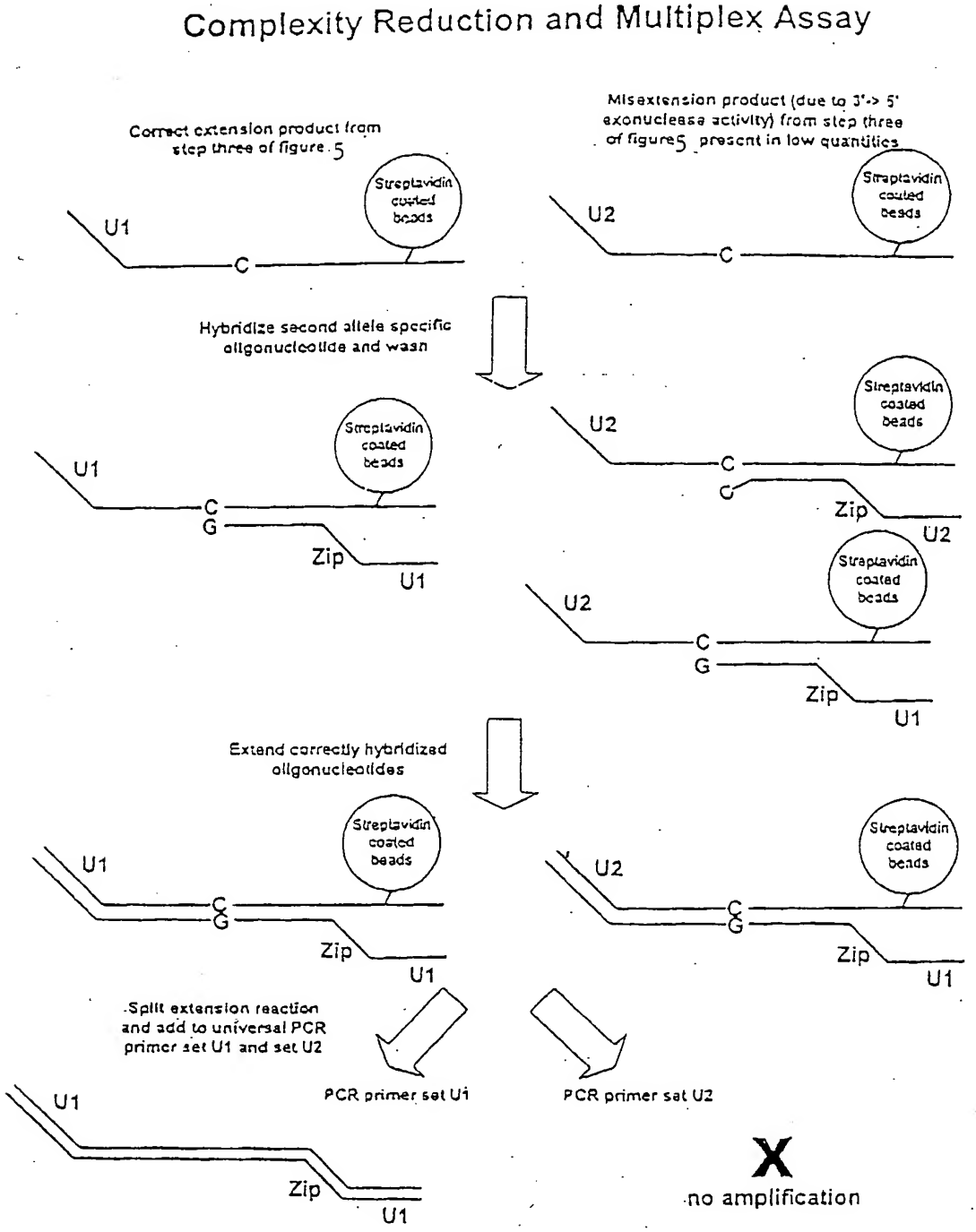

Complexity Reduction and Multiplex Assay
Correct extension product from step three of figure 5
Misextension product (due to 3'-> 5' exonuclease activity) from step three of figure5 present in low quantities
Streptavidin coated beads
U1
U2
C
Hybridize second allele specific oligonucleotide and wash
G
Zip
Extend correctly hybridized oligonucleotides
Split extension reaction and add to universal PCR primer set U1 and set U2
PCR primer set U1
PCR primer set U2
X
no amplification

FIGURE 6

Solid Phase Locus-Specific Primer Extension

Starting material is immobilized, single stranded universal PCR product. There are several ways to generate this.

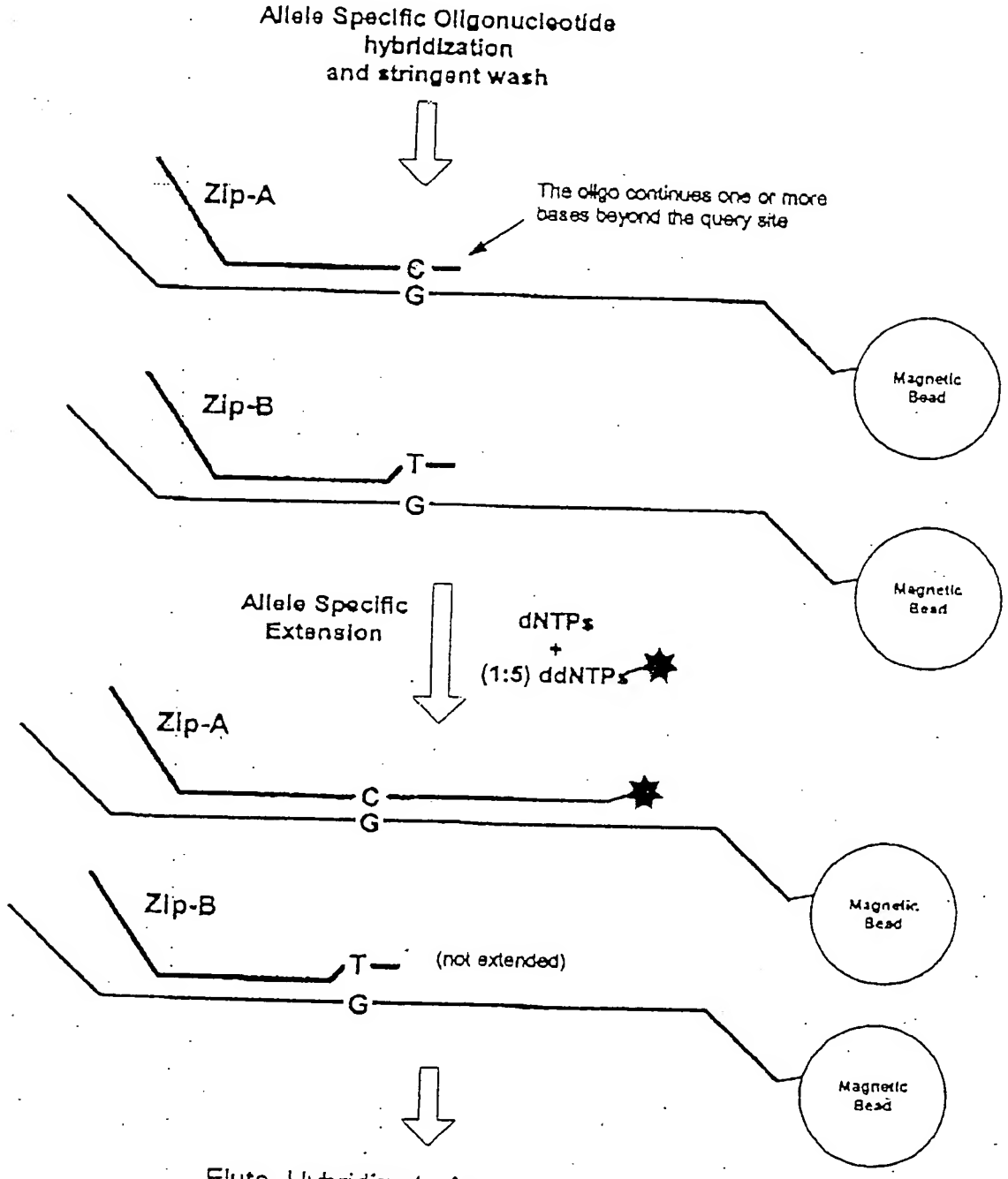


FIGURE 7

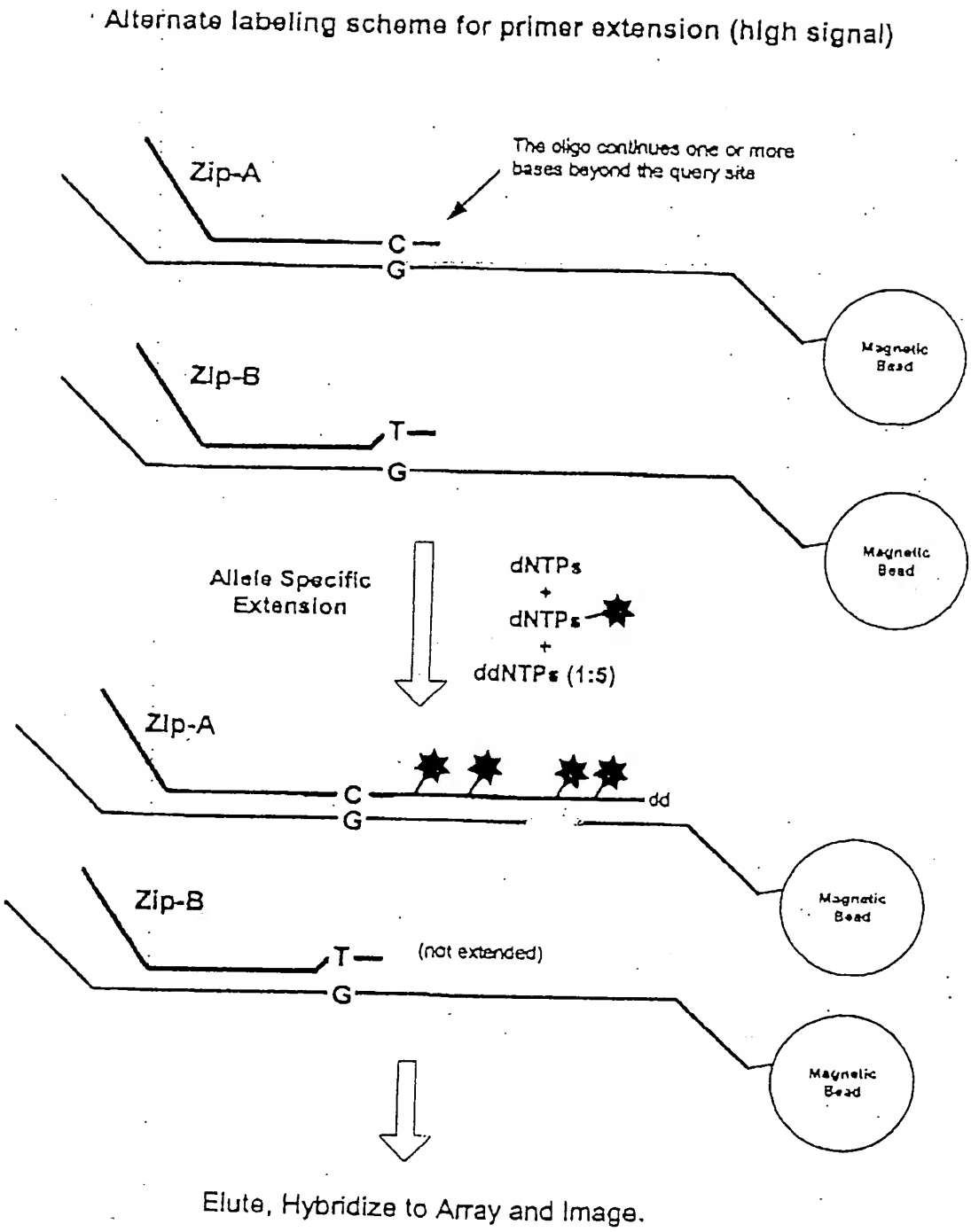

Alternate labeling scheme for primer extension (high signal)
Zip-A
The oligo continues one or more bases beyond the query site
C
G
Magnetic Bead
Zip-B
T
G
Magnetic Bead
Allele Specific Extension
dNTPs
+
dNTPs
+
ddNTPs (1:5)
Zip-A
C
G
dd
Magnetic Bead
Zip-B
T
(not extended)
G
Magnetic Bead
Elute, Hybridize to Array and Image.

FIGURE 8

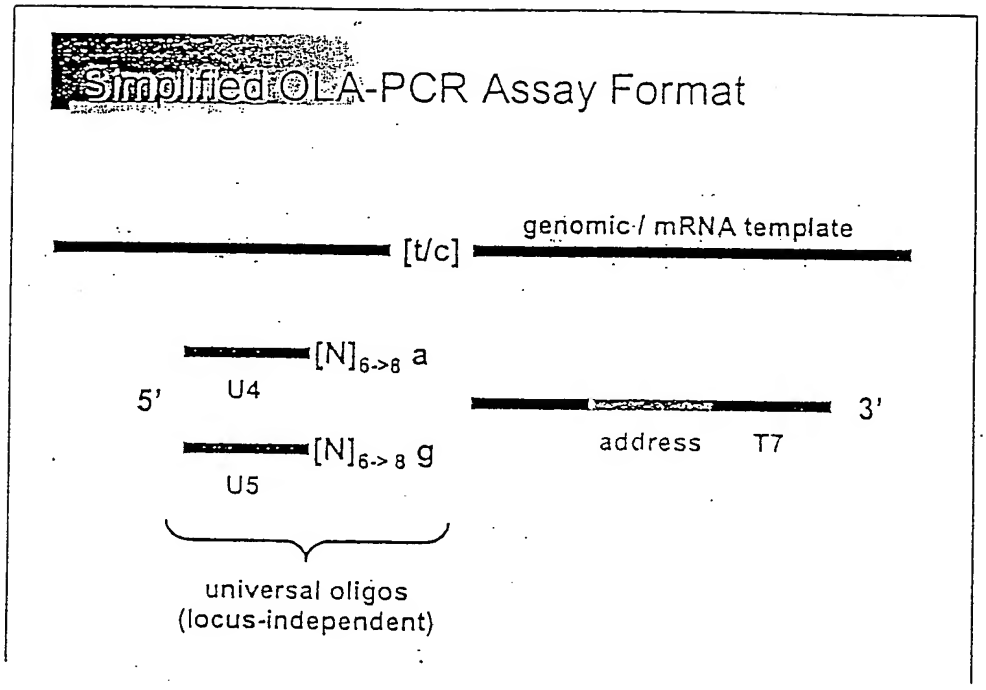

Simplified OLA-PCR Assay Format
genomic / mRNA template
[t/c]
[N]6->8 a
5'
U4
3'
address
T7
[N]6->8 g
U5
universal oligos
(locus-independent)

FIGURE 9

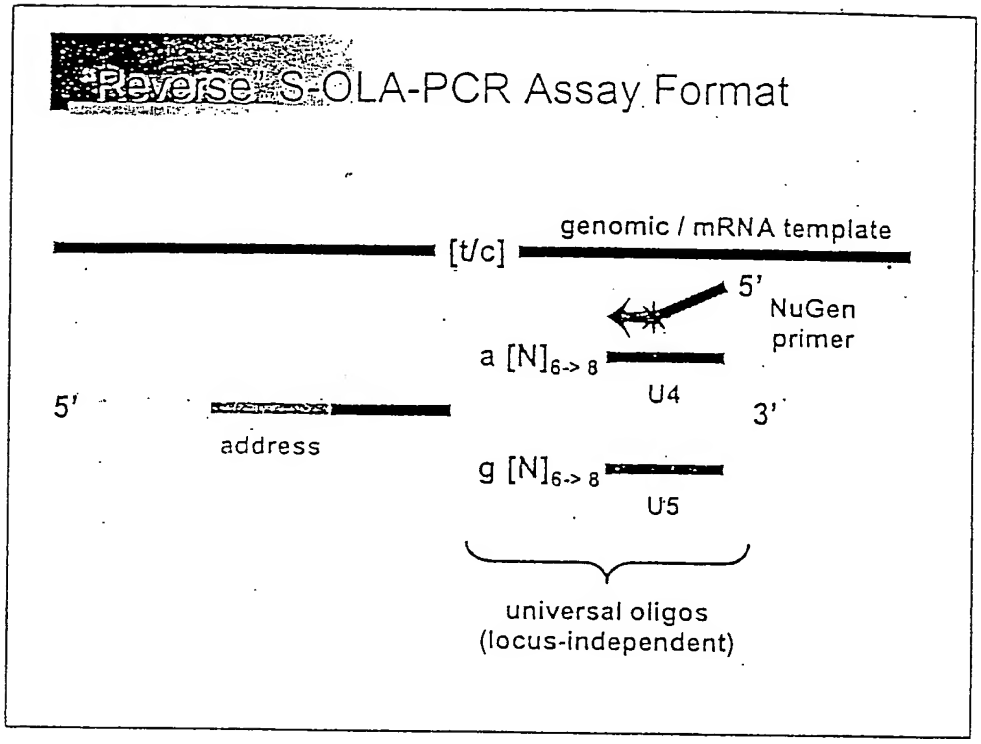

"Reverse" S-OLA-PCR Assay Format
genomic / mRNA template
[t/c]
5'
NuGen
primer
a [N]6-> 8
U4
5'
3'
address
g [N]6-> 8
U5
universal oligos
(locus-independent)

FIGURE 10

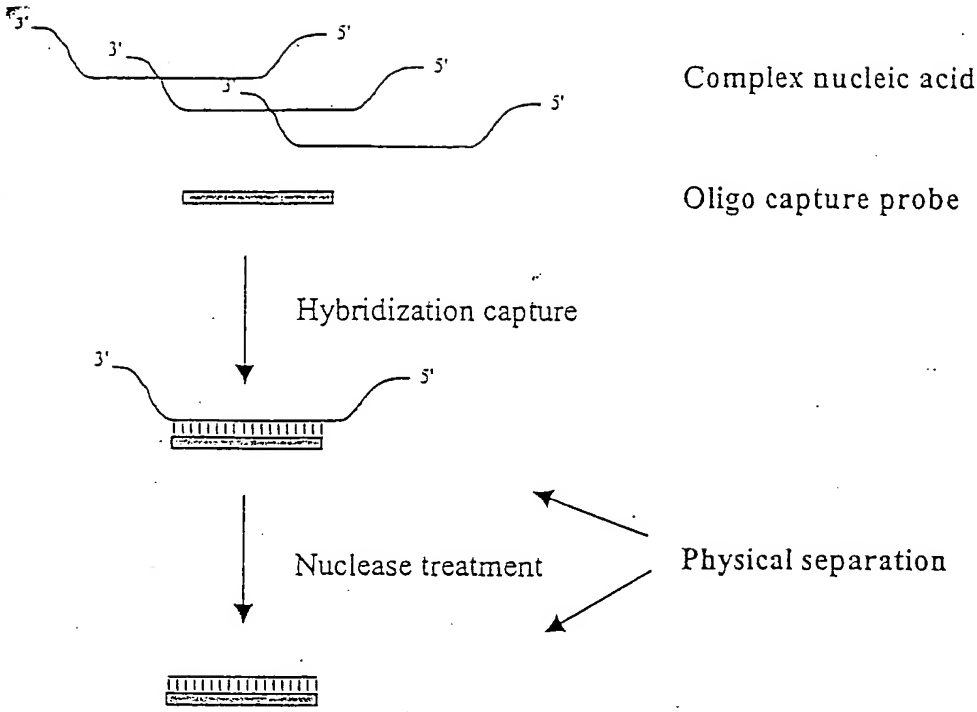

3'
5'
Complex nucleic acid mixture
Oligo capture probe
Hybridization capture
Nuclease treatment
Physical separation

FIGURE 11

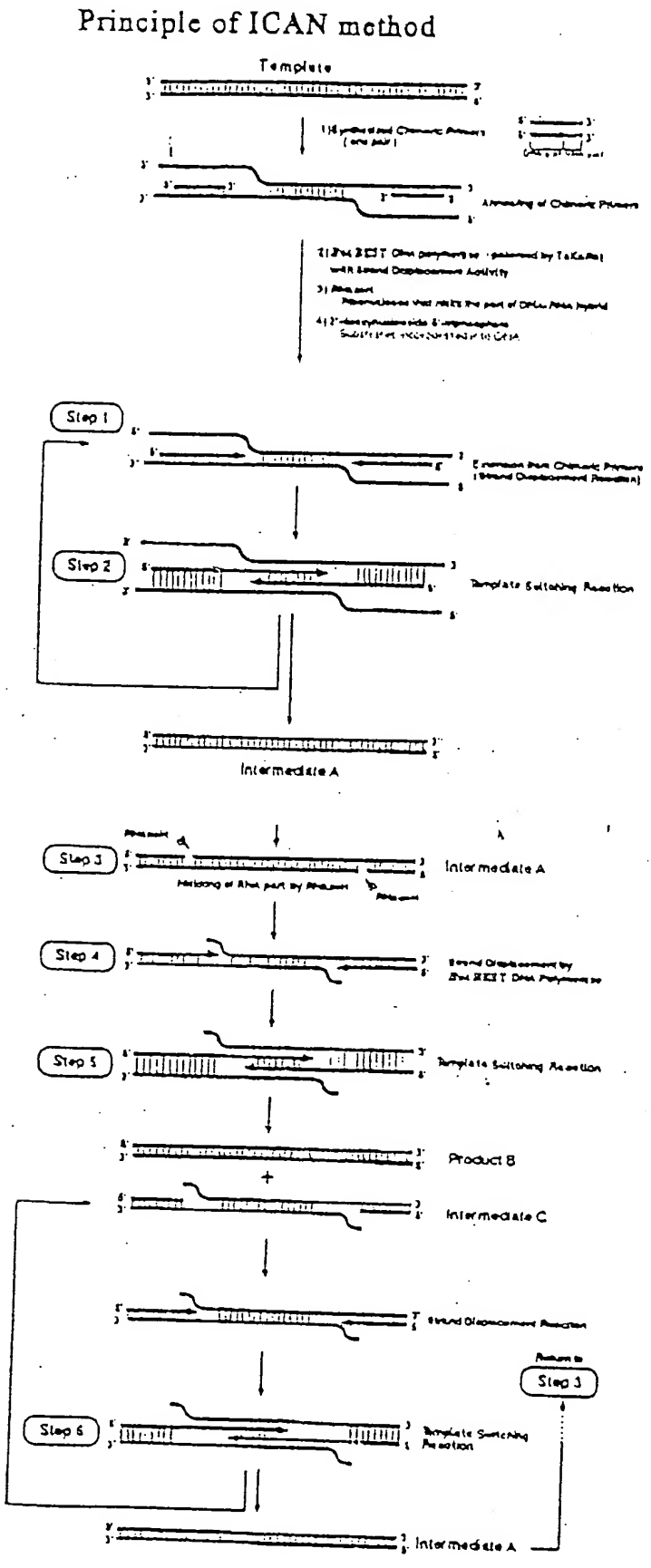

Principle of ICAN method
Template
Step 1
Step 2
Intermediate A
Step 3
Intermediate A
Step 4
Step 5
Product B
Intermediate C
Step 6
Intermediate A

FIGURE 12

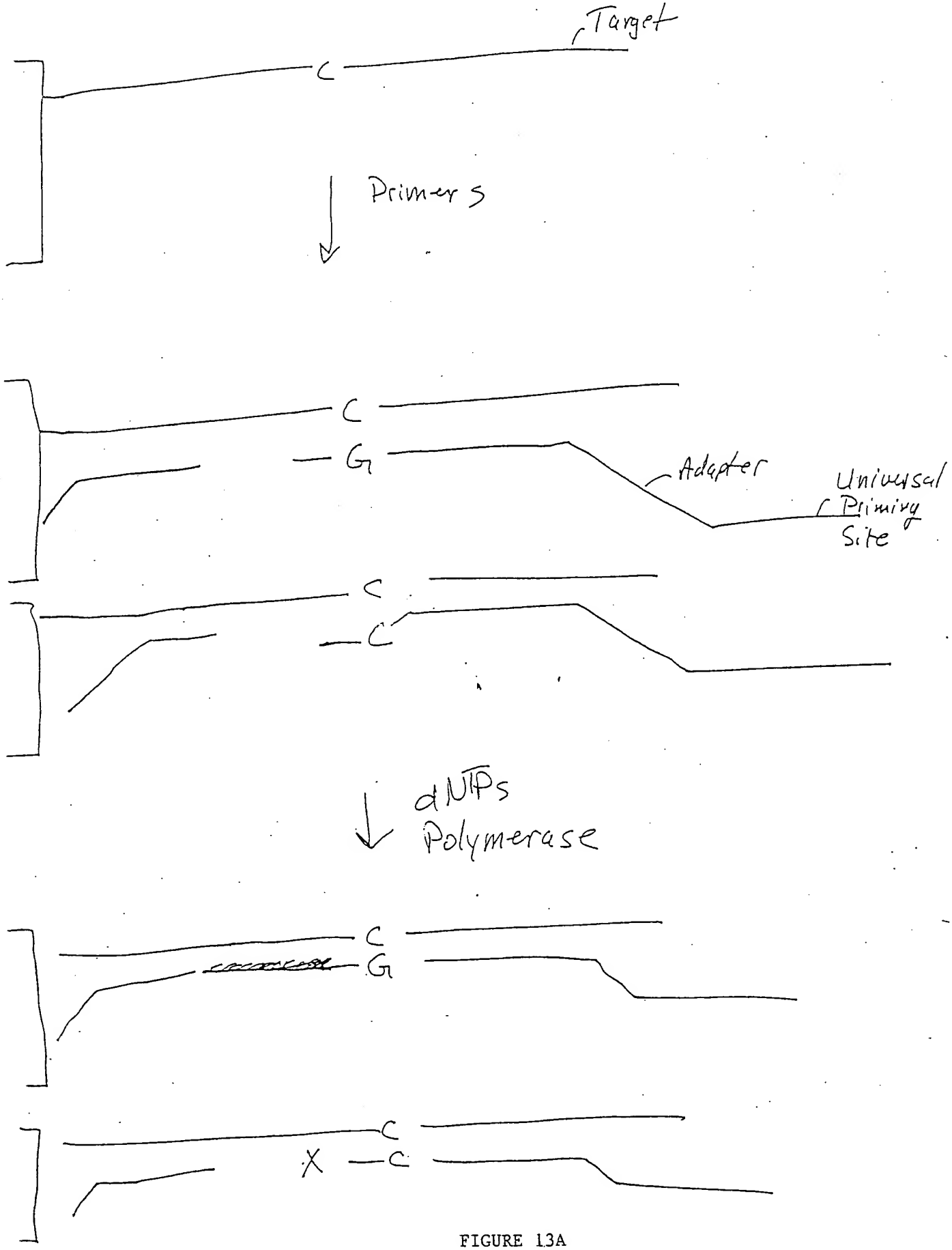

Target
C
Primers
C
G
Adapter
Universal
Priming
Site
C
C
dNTPs
Polymerase
C
G
C
X
C

FIGURE 13A

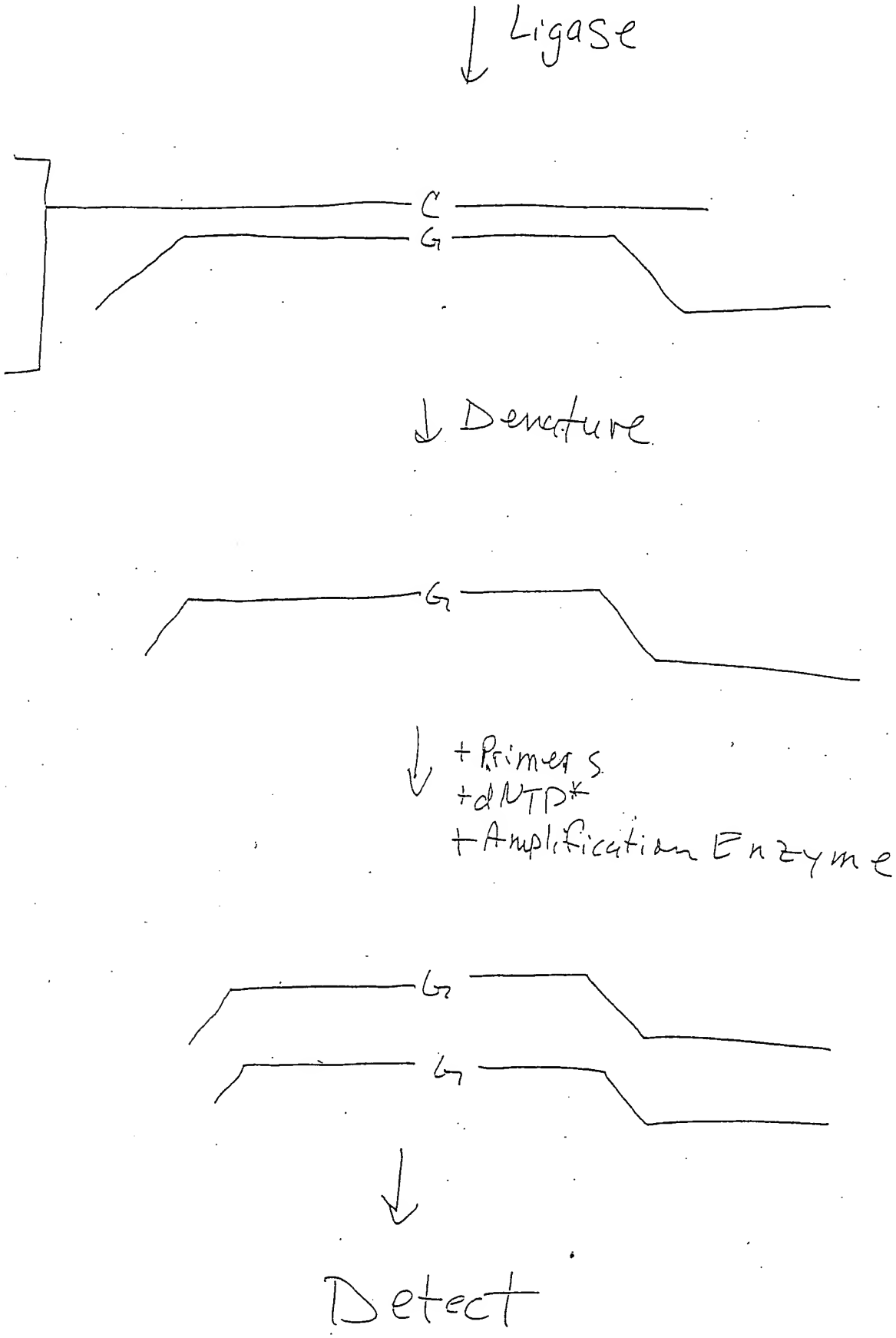

Ligase
C
G
Denature
G
+Primers
+dNTP*
+Amplification Enzyme
G
G
Detect

FIGURE 13B

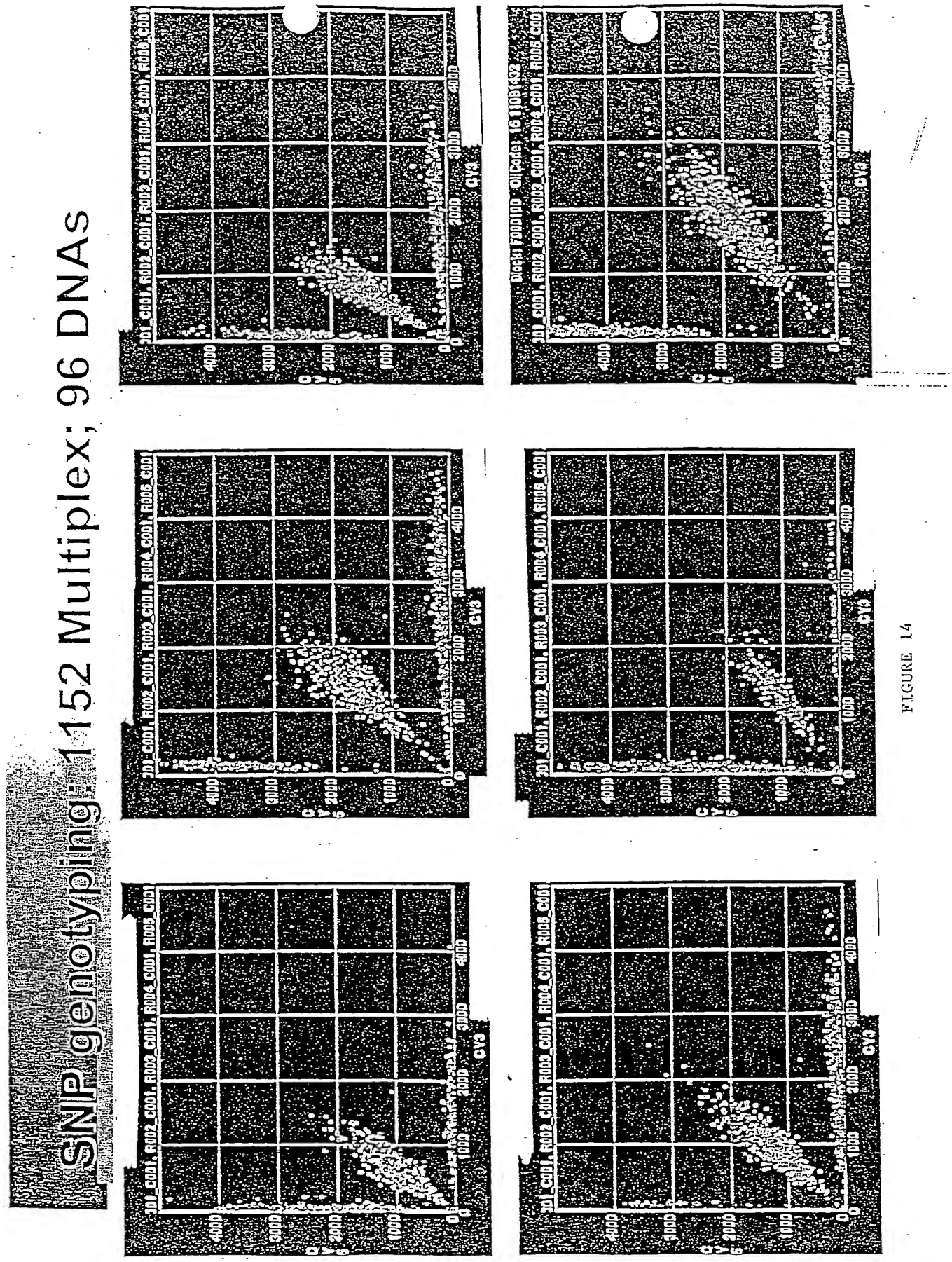


FIGURE 14

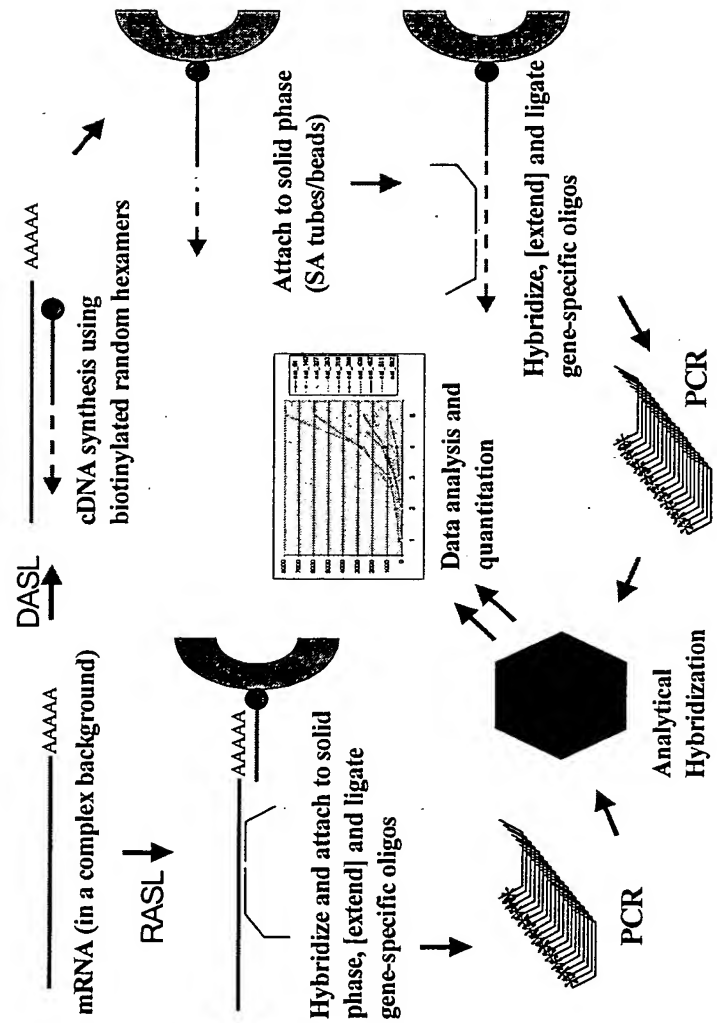
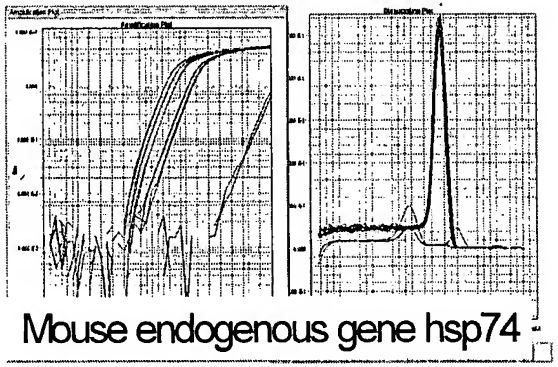
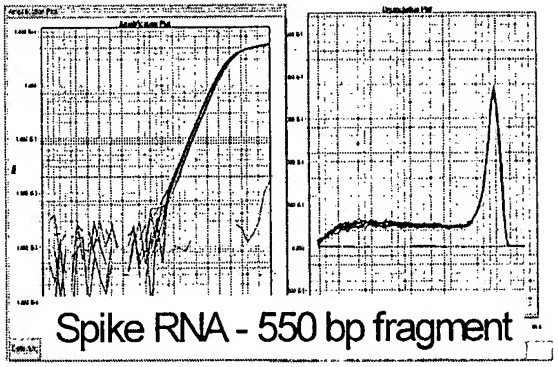
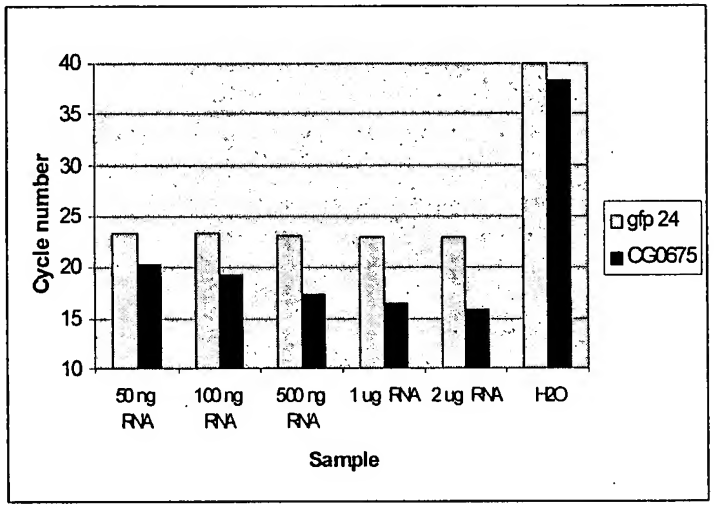

AAAAA
mRNA (in a complex background)
RASL
AAAAA
Hybridize and attach to solid phase, [extend] and ligate gene-specific oligos
PCR
Analytical Hybridization
Data analysis and quantitation
DASL
AAAAA
cDNA synthesis using biotinylated random hexamers
Attach to solid phase (SA tubes/beads)
Hybridize, [extend] and ligate gene-specific oligos
PCR

FIGURE 15

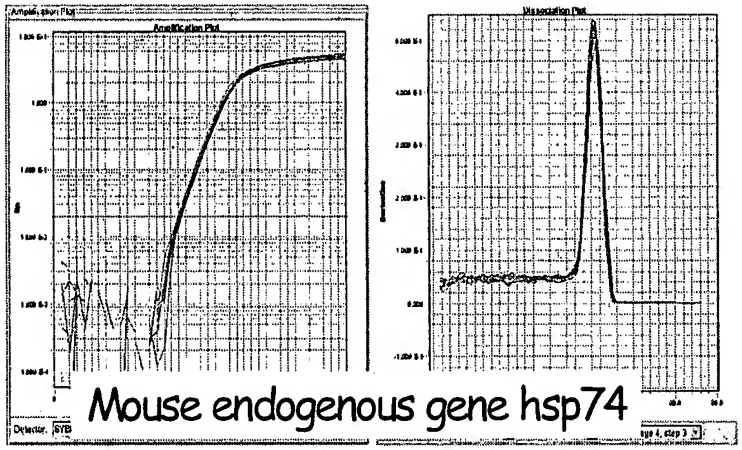




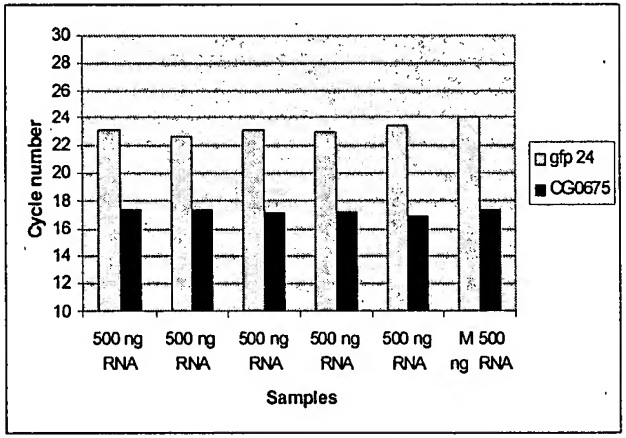


- Total RNA input varied from 50 ng to 2 ug.
- Spike RNAs were added at constant concentration - 10^6 molecules pro sample.
- cDNA synthesis on robot was successful!

FIGURE 16



		100 ng	500 ng
gfp 24	Avg	23.57	23.23
	SD	0.42	0.48
	CV	0.02	0.02
CG0675	Avg	19.78	17.20
	SD	0.66	0.15
	CV	0.03	0.01



- cDNA synthesis on the robot was reproducible and as good as in the manual reaction (at least for the tested genes).

FIGURE 17

Title: Multiplex Nucleic Acid Reactions
Inventors: Chee et al.
Filing Date: Herewith
Attorney Client-Matter No.:67234-015
(858-535-9001)

	pool 1	pool 2	pool 3	pool 4	pool 5	pool 6	pool 7	pool 8
cat	0.00E+00	1.00E+04	3.00E+04	1.00E+05	3.00E+05	1.00E+06	3.00E+06	1.00E+07
cre	1.00E+04	3.00E+04	1.00E+05	3.00E+05	1.00E+06	3.00E+06	1.00E+07	0.00E+00
E1A	3.00E+04	1.00E+05	3.00E+05	1.00E+06	3.00E+06	1.00E+07	0.00E+00	1.00E+04
GFP	1.00E+05	3.00E+05	1.00E+06	3.00E+06	1.00E+07	0.00E+00	1.00E+04	3.00E+04
gus	3.00E+05	1.00E+06	3.00E+06	1.00E+07	0.00E+00	1.00E+04	3.00E+04	1.00E+05
lacZ	1.00E+06	3.00E+06	1.00E+07	0.00E+00	1.00E+04	3.00E+04	1.00E+05	3.00E+05
luc	3.00E+06	1.00E+07	0.00E+00	1.00E+04	3.00E+04	1.00E+05	3.00E+05	1.00E+06
neo	1.00E+07	0.00E+00	1.00E+04	3.00E+04	1.00E+05	3.00E+05	1.00E+06	3.00E+06
→ bla	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05
→ GST	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05	3.00E+05

FIGURE 18

Title: Multiplex Nucleic Acid Reactions
Inventors: Chee et al.
Filing Date: Herewith
Attorney Client-Matter No.:67234-015
(858-535-9001)

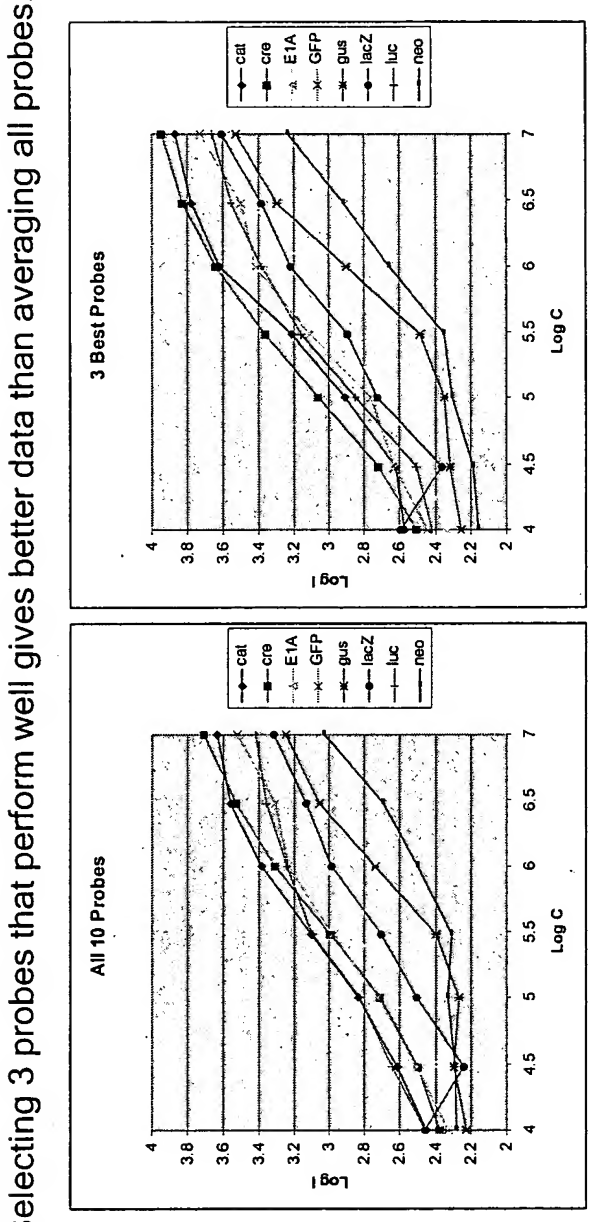

Selecting 3 probes that perform well gives better data than averaging all probes.
All 10 Probes
3 Best Probes
cat
cre
E1A
GFP
gus
lacZ
luc
neo
Log I
Log C
Matrix 4, 238-plex, 100 ng total RNA background

FIGURE 19

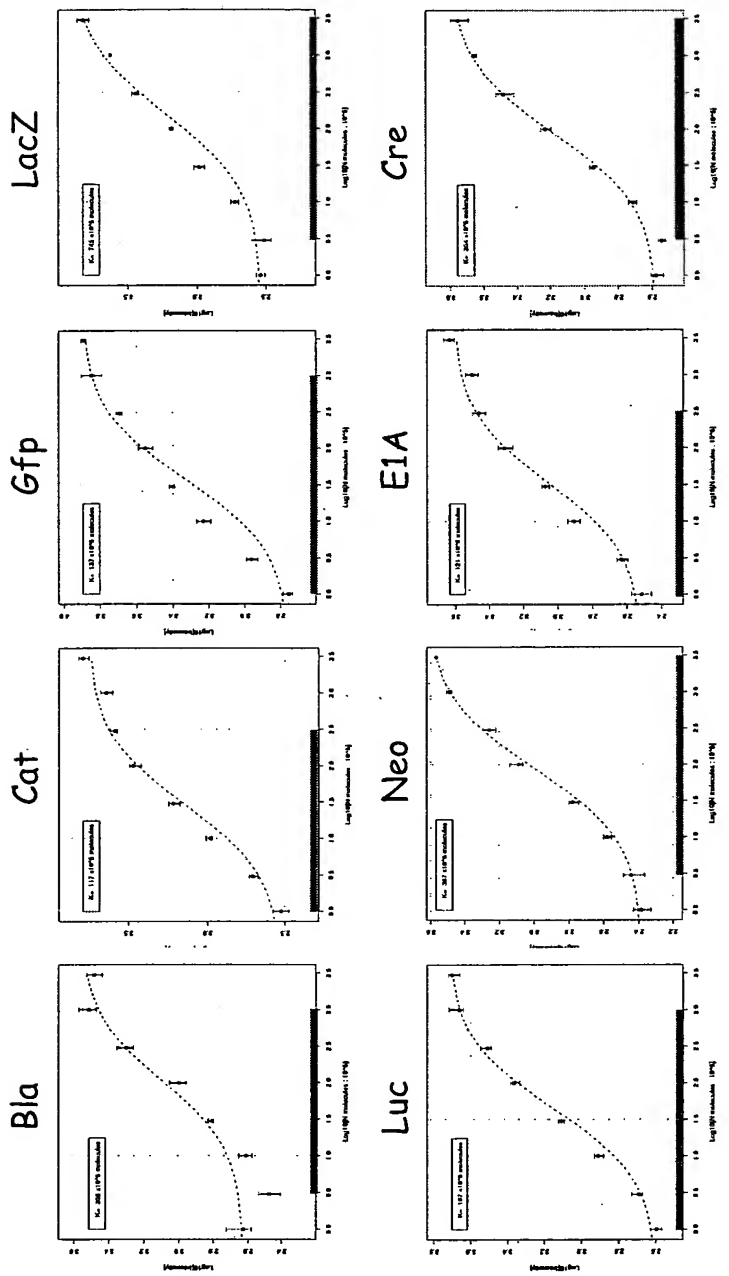

LacZ
Gfp
Cat
Bla
Cre
E1A
Neo
Luc
3 fold detection range
●Error bars represent the range of intensities of 4 replicates.

FIGURE 20

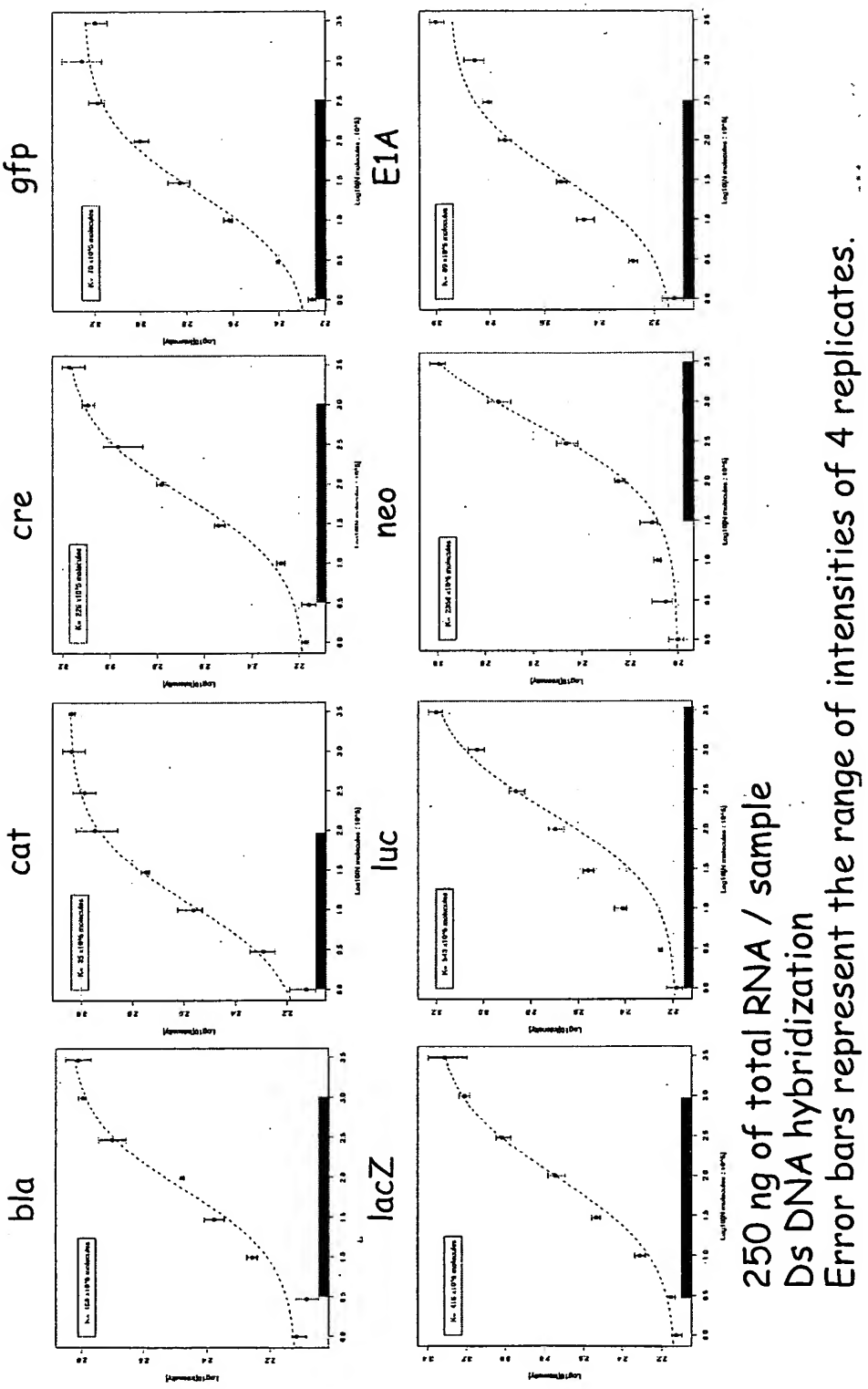
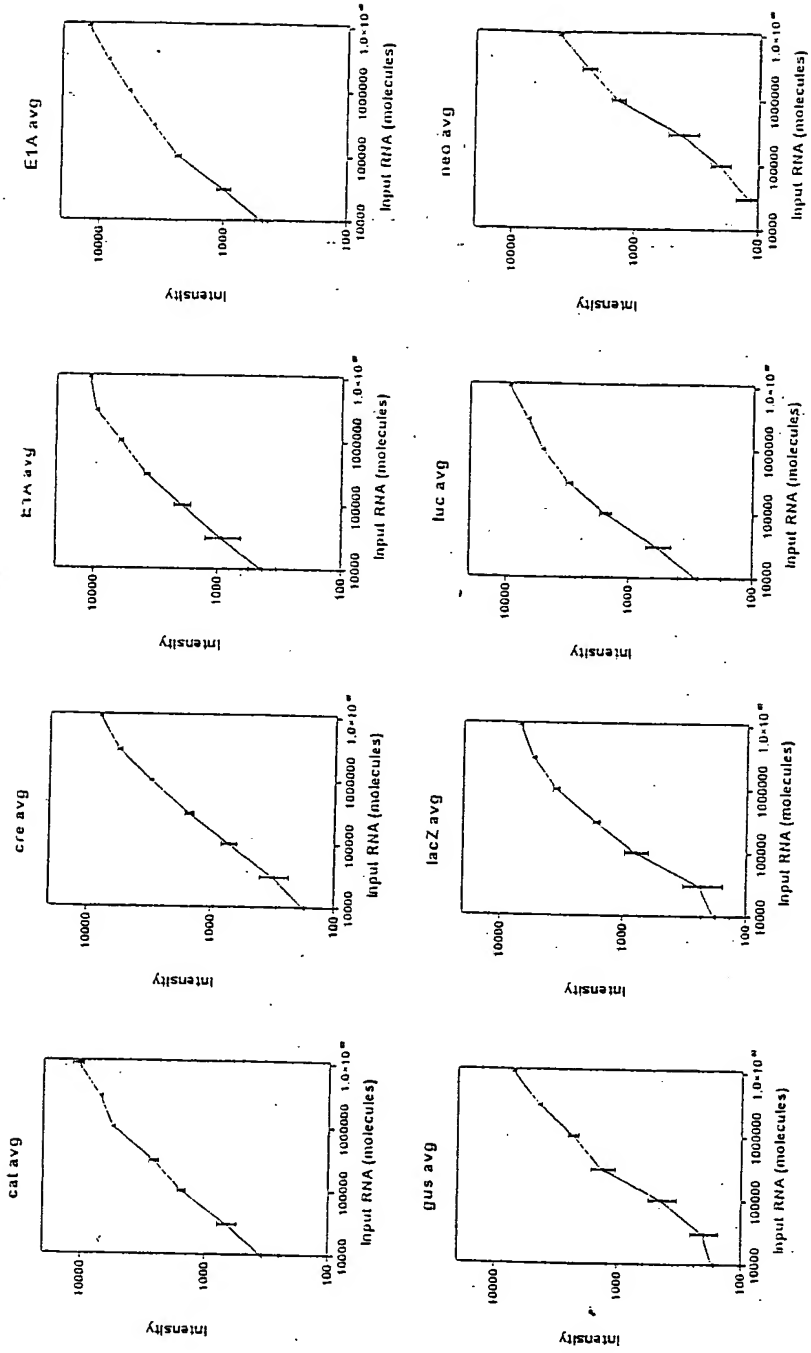

bla
cat
cre
gfp
lacZ
luc
neo
E1A
250 ng of total RNA / sample
Ds DNA hybridization
Error bars represent the range of intensities of 4 replicates.

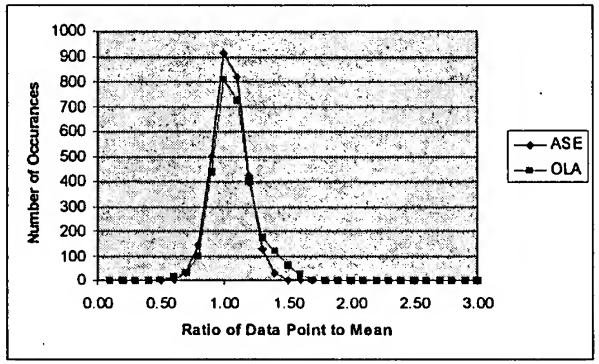
FIGURE 21



- 100 ng total RNA background, 12 replicates, 238-plex.
- all pre-PCR and post-PCR processes identical to SciOps including single stranded product hybridization to arrays.
- Dynamic range: 2.5 - 3 logs; Precision: better than 3 fold change.

FIGURE 22

(500 ng input RNA)



● 100.0% data points among 4 replicates within 2 fold change

◎ 99.8% data points among 4 replicates within 2 fold change

FIGURE 23

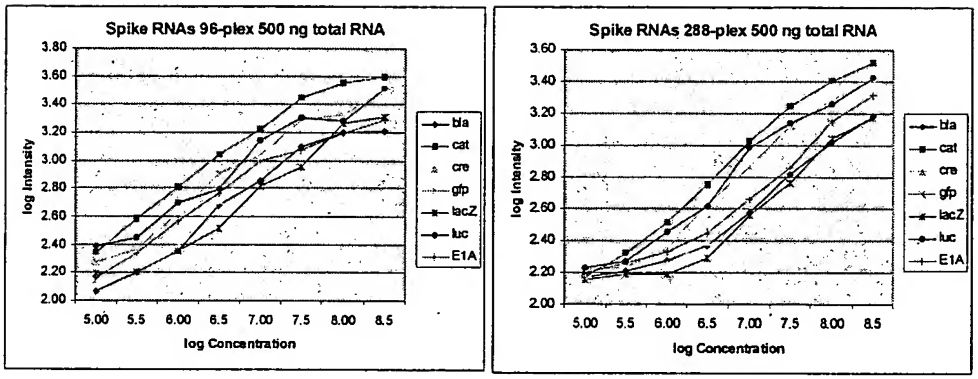
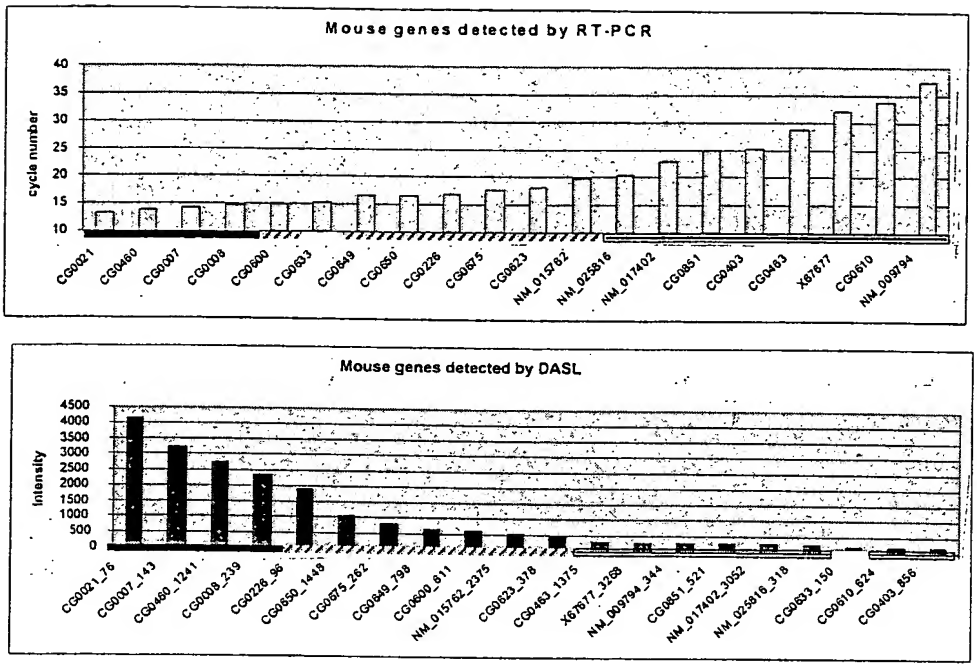

Spike RNAs 96-plex 500 ng total RNA
log Intensity
log Concentration
bla
cat
cre
gfp
lacZ
luc
E1A
Spike RNAs 288-plex 500 ng total RNA
log Intensity
log Concentration
bla
cat
cre
gfp
lacZ
luc
E1A

FIGURE 24


Mouse genes detected by RT-PCR
cycle number
40
35
30
25
20
15
10
CG0021
CG0460
CG0007
CG0008
CG0600
CG0633
CG0649
CG0650
CG0228
CG0675
CG0623
NM_015762
NM_025816
NM_017402
CG0851
CG0403
CG0483
X67677
CG0610
NM_009794

Mouse genes detected by DASL
Intensity
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CG0021_76
CG0007_143
CG0460_1241
CG0008_239
CG0228_96
CG0650_1449
CG0675_262
CG0649_798
CG0600_811
NM_015762_2375
CG0623_378
CG0483_1375
X67677_3288
NM_009794_344
CG0851_521
NM_017402_3052
NM_025816_318
CG0633_150
CG0610_824
CG0403_856

FIGURE 25

How to Handle Genes Expressed at Different Levels?

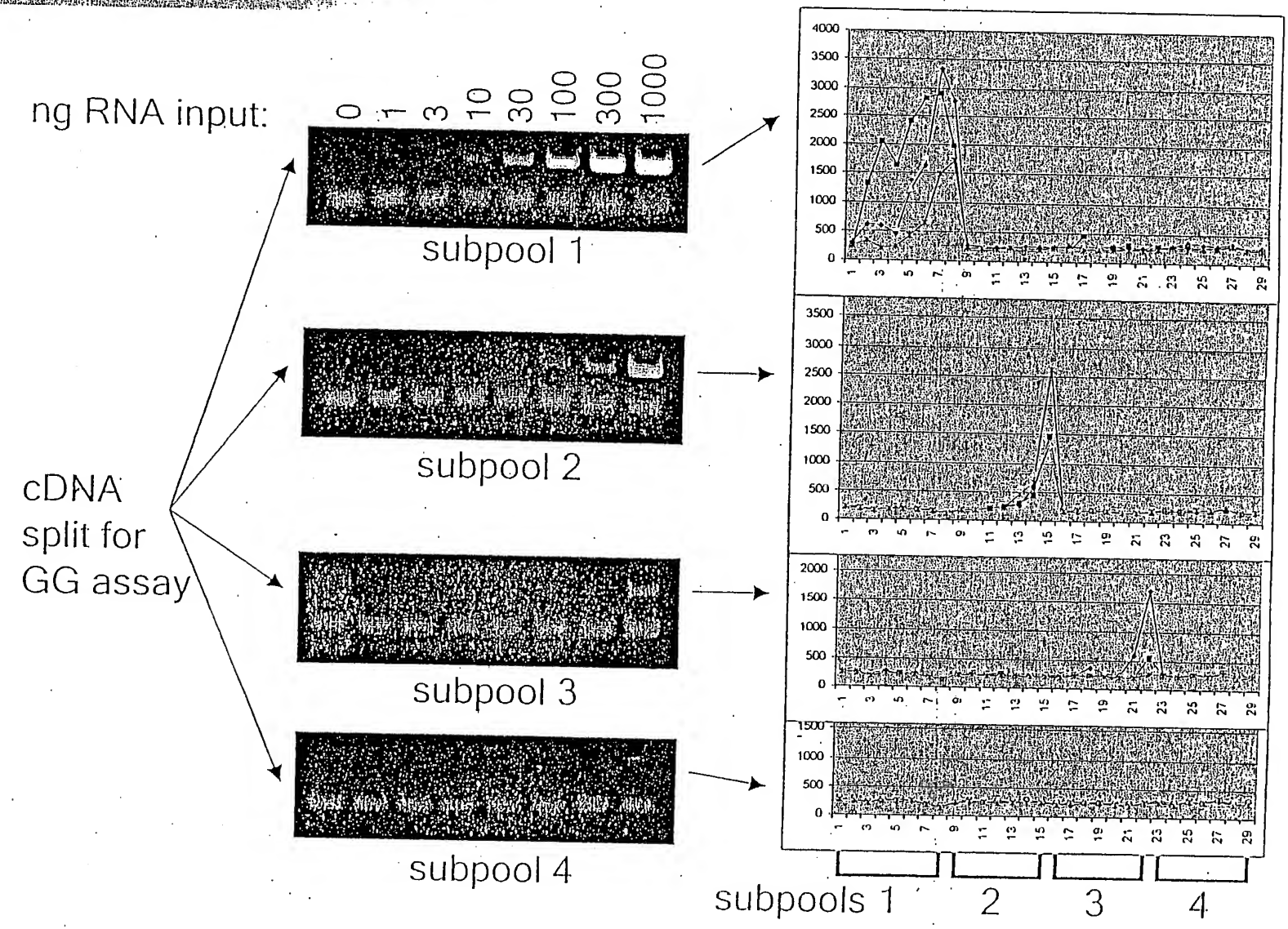


FIGURE 26

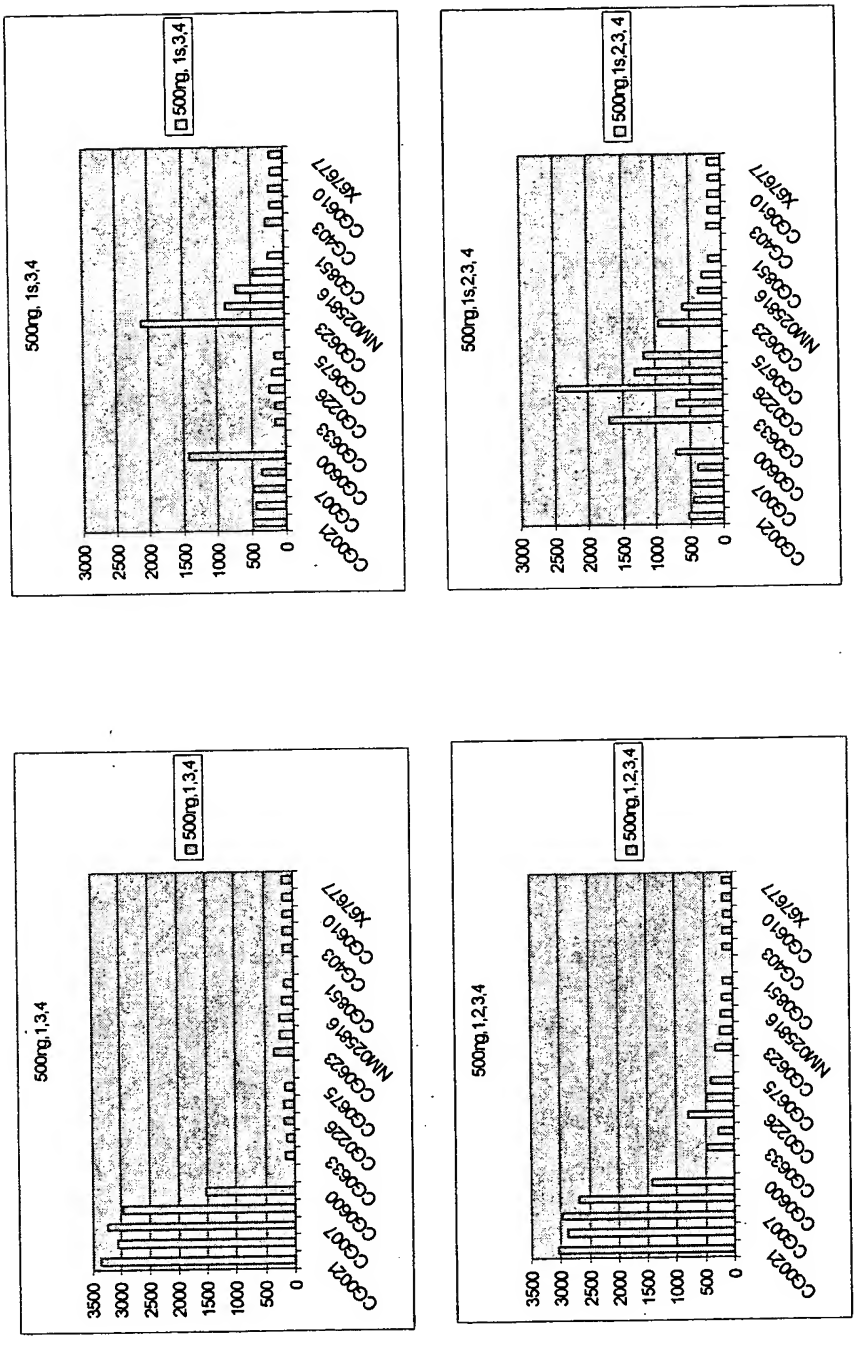

500ng, 1s,3,4
500ng,1s,2,3, 4
500ng,1,3,4
500ng,1,2,3,4
500ng, 1s,3,4
500ng,1s,2,3, 4
500ng,1,3,4
500ng,1,2,3,4
3000
2500
2000
1500
1000
500
0
3500
CG002A
CG007
CG600
CG633
CG228
CG675
CG623
NM025816
CG851
CG403
CG610
X67677

FIGURE 27